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The cytotoxic T lymphocyte antigen-4 is a major Graves' disease locus

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Graves' disease (GD) is an autoimmune thyroid disorder that is inherited as a complex trait. We have genotyped 77 affected sib-pairs with autoimmune thyroid disease for eight polymorphic markers spanning the cytotoxic T lymphocyte antigen-4 (*CTLA-4*) region of chromosome 2q31–q33, and for five markers spanning the major histocompatibility complex (*MHC*) region of chromosome 6p21. Non-parametric analysis showed linkage of GD to the *CTLA-4* region with a peak non-parametric linkage (NPL) score of 3.43 ($P = 0.0004$) at the marker *D2S117*. The proportion of affected full-sibs sharing zero alleles (z0) reached a minimum of 0.113 close to *D2S117*, giving a locus-specific λ_s for this region of 2.2. Families with brother–sister sib-pairs showed a peak NPL of 3.46 ($P = 0.0003$, $\lambda_s > 10$) at *D2S117*, compared with 2.00 ($P = 0.02$, $\lambda_s = 1.9$) in the families with only affected females, suggesting a stronger influence in families with affected males. Association between GD and the G allele of the Thr17Ala polymorphism within the *CTLA-4* gene (*CTLA4A/G*) was observed using unaffected sib controls ($P = 0.005$). Lesser evidence for linkage was found at the *MHC* locus, with a peak NPL score of 1.95 ($P = 0.026$), between the markers *D6S273* and *TNFα*. We demonstrate that the *CTLA-4* locus ($\lambda_s = 2.2$) and the *MHC* locus ($\lambda_s = 1.6$) together confer ~50% of the inherited susceptibility to GD disease in our population.

INTRODUCTION

Graves' disease (GD) is a common organ-specific autoimmune disorder, which is characterized by thyroid hormone oversecretion, diffuse goitre and specific orbital complications (termed thyroid-associated orbitopathy: TAO). GD affects 0.4–0.8% of the female population over a life time (1–3), and has a concordance rate in monozygotic twins of 20–30% compared with 5–7% in dizygotic twins or female sibs (2–4). Familial risk studies combined with local population prevalence data have been used to estimate that the excess risk of GD to a female sib of a GD proband (λ_s) is between 10 and 15 (2,3). In common with other autoimmune disorders (5–9), GD is likely to have a complex genetic basis, with several different genes each contributing in various degrees to the inherited susceptibility. Furthermore, autoimmune hypothyroidism (AH), which is the other common manifestation of thyroid autoimmunity, occurs with an increased frequency in GD kindreds, suggesting that both forms of autoimmune thyroid disease (AITD) could share some susceptibility alleles (10,11). Similarly, there is an excess prevalence of GD amongst subjects with type 1 diabetes mellitus (IDDM) and their relatives, suggesting that GD and IDDM could also share susceptibility alleles (10–12).

Population-based case–control studies have shown a consistent association of GD with HLA-DR3-carrying major histocompatibility complex (MHC) haplotypes (*DRB1*0304–DQB1*0201–DQA1*0501*) in Caucasian populations (2,10,13–15); however, these MHC allelic associations may be different from those of AH (10). Evidence for linkage of GD to *MHC* has also been found in some populations (10,15–17), but this has been difficult to reproduce (18,19). Other candidate susceptibility loci, including the thyrotropin receptor, immunoglobulin heavy chain (*Gm*), interleukin 1 receptor antagonist, T cell antigen receptor β and the thyroid hormone receptor β gene have been found to be associated with GD in some populations studied, but not in others (16,20–25). Recently, evidence for varying degrees of genetic linkage have been reported between AITD and markers on the long arms of

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Table 1. Phenotypes of affected sib-pairs with autoimmune thyroid disease

	Graves' disease only (GD–GD)	Mixed Graves' and AH (GD–AH)[a]	All autoimmune thyroid disease
Full sib-pairs	66	6	72
Half sib-pairs	5	0	5
Total	71	6	77

[a]Families were selected on the basis of two affected GD sibs. GD–AH sib-pairs make up additional members of the same families.

chromosomes 14 (14q31) and 20 (20q11) and on the X chromosome (Xq21) (19,26,27). These studies await confirmation in different populations.

The cytotoxic T lymphocyte antigen-4 (CTLA-4) is a co-stimulatory molecule that is a key negative regulator of T cell function (28). The *CTLA-4* gene lies on chromosome 2q33, and recent studies have demonstrated linkage and association of IDDM with markers on 2q31–q33 (designated *IDDM7* and *IDDM12*) (29–32). Transmission disequilibrium at alleles of two *CTLA-4* polymorphisms defines the *IDDM12* region (30,31), and the same alleles have been associated with GD and AH in case–control studies (32–36). However, recent genetic linkage studies have failed to confirm *CTLA-4* as a GD susceptibility locus (19,37). In this study, we have taken an affected sib-pair approach to examine the *CTLA-4* region of chromosome 2q31–q33 and the *MHC* region of 6p21 for evidence of linkage to GD.

RESULTS

Chromosome 2q31–q33 linkage analysis.

We examined the cohort of 77 affected AITD sib-pairs (Table 1) for linkage to eight polymorphic markers over a 30 cM region of chromosome 2q31–q33, which encompassed the *IDDM7* and *CTLA-4* (*IDDM12*) regions. Non-parametric analysis with the GENEHUNTER package showed a broad region of excess allele sharing (up to 65%) amongst affected sibs. When all subjects with AITD were designated as affected, the peak multipoint non-parametric linkage (NPL) score was 3.43 ($P = 0.0004$) at the marker *D2S117*, which is close to the *CTLA-4* region (Fig. 1). Designation of only GD cases as affected (71 sib-pairs) showed a peak NPL score of 3.07 ($P = 0.001$; Fig. 1), suggesting homogeneity between GD and AH at this locus. The proportion of the 72 full-sibs with AITD sharing zero alleles (z0) reached a minimum of 0.113 close to *D2S117*, suggesting that the locus-specific λ_s for this region is 2.2. The 24 families with affected AITD males (brother–sister sib-pairs) showed a peak NPL of 3.46 ($P = 0.0003$, $z0 < 1 \times 10^{-4}$, $\lambda_s > 10$), compared with 2.00 ($P = 0.02$, $z0 = 0.132$, $\lambda_s = 1.9$) in the 40 families with only affected females, suggesting that this locus has a much stronger influence in families with affected males. An intrafamilial association analysis, using unaffected sibs as controls, showed an excess of the G allele at the diallelic *CTLA4A/G* polymorphism (Thr17Ala) ($P = 0.005$), and of the 112 mobility unit (mu) allele of *CTLA4[AT]n* ($P = 0.02$) in GD probands (Table 2). The odds ratio (OR) for the G allele at *CTLA4A/G* in GD probands was 2.01 (95% CI 1.13–4.55; $P = 0.047$) compared with unaffected sibs, and for the GG genotype (versus AG and AA) was 4.56 (95% CI 1.06–22.02; $P = 0.028$). The OR for the G allele in affected GD males compared with affected female sibs was 1.55 (95% CI 0.68–3.52; $P = 0.2$).

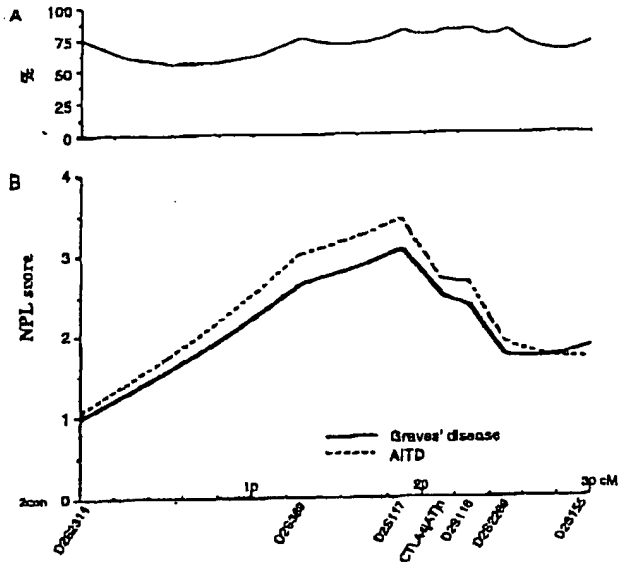

Figure 1. Multipoint linkage analysis of eight markers on chromosome 2q31–q33. (A) Percentage information content over the marker map. (B) The NPL score obtained by 'scoring all' affected subjects with Graves' disease (solid line) and AITD (dashed line) using the GENEHUNTER package is shown, against the marker map on the *x*-axis. The diallelic polymorphism *CTLA4A/G* is at the same map position as *CTLA4[AT]n*. The peak NPL score of 3.43 occurs at the marker *D2S117*.

Analysis of chromosome 2q31–q33 linkage conditioned for the *CTLA4A/G* genotype

Families were weighted according to the *CTLA4A/G* genotype of the proband, and non-parametric linkage analysis was carried out with AITD subjects designated as affected. The maximum evidence for linkage came from the families whose proband carried the AG genotype, with a peak NPL score of 2.76 occurring at *D2S117* (Table 3). Lesser evidence for linkage was found in the families with AA- or GG-carrying probands, with peak NPL scores of 2.14 and 1.36, respectively (Table 3). Despite the association between GD and the G allele of *CTLA4A/G*, we find no evidence that the linkage is confined to families whose probands carry this allele.

MHC linkage analysis

Examination of five microsatellite markers spanning a 23 cM region of chromosome 6p21 encompassing the *MHC* locus showed a modest increase in allele sharing (up to 58%) amongst the 71 affected GD sib-pairs, with a peak multipoint NPL score of 1.95 ($P = 0.026$) occurring at the markers *D6S273* and *TNFα* (Fig. 2). Scoring all subjects with AITD as being affected led to a decrease in the peak NPL score (1.40), in keeping with GD and AH having different *MHC* susceptibility alleles. The minimum z0 value for the 66 full sib-pairs with GD was 0.161 at the

Table 2. Pair-wise association between affected (GD) and unaffected sib controls at linked chromosome 2q31–q33 markers

Marker	Allele	Graves' probands[a]	Unaffected sibs[a]	P-value[b]
D2S389	203 mu	10/12	5/17	NS
D2S117	206 mu	8/10	3/15	NS
CTLA4A/G	G	20/6	10/16	0.005
CTLA4[AT]n	112 mu	14/4	7/11	0.020
D2S116	150 mu	9/7	3/13	NS

[a]Number of occurrences of candidate alleles/non-occurrences (45).
[b]Fisher's exact test, corrected for multiple allelic comparisons.

Table 3. Analysis of chromosome 2q31–q33 linkage, conditioned for the *CTLA4A/G* genotype of the proband

Genotypes of probands	Peak NPL score	P-value	No. of families
GG	1.36	0.087	13
AG	2.76	0.003	30
AA	2.14	0.016	21
GG or AG	3.05	0.001	43
AG or AA	3.28	0.0006	51
All families	3.43	0.0004	64

marker *TNFα*, suggesting that the locus-specific λ_s for the *MHC* region is 1.6.

Analysis of *CTLA-4* linkage conditioned for MHC haplotype sharing

Families were subdivided according to identity by descent (IBD) haplotype sharing at the *TNFα* locus, and the linkage to the chromosome 2 markers was re-examined. The peak NPL score for sib-pairs sharing two *MHC* haplotypes was 2.38 (P = 0.008), compared with 2.19 (P = 0.015) for sibs sharing 1 or 0 *MHC* haplotypes.

DISCUSSION

We have demonstrated, for the first time, unequivocal evidence (P = 0.0004) for linkage of GD to the *D2S117* region of 2q33 (Fig. 1), with a locus-specific λ_s of 2.2. The type 1 diabetes loci *IDDM7* and the *CTLA-4* gene (*IDDM12*) are close to the linked region, and it is possible that both may contain susceptibility polymorphisms that contribute to the linkage we observe. Our association analysis, which shows evidence for intrafamilial allelic association at the two *CTLA-4* markers (*CTLA4A/G* and *CTLA4[AT]n*) but not at other loci within this region (Table 2), would suggest that a susceptibility polymorphism(s) lies at, or close to, the *CTLA-4* locus. However, subgroup analysis shows that the evidence for linkage to this region is not confined to families with G allele-carrying *CTLA4A/G* genotypes, such that the effect of this polymorphism alone is not sufficient to explain the observed linkage. The strength of the linkage that we observed in the 24 families with affected male GD members (P = 0.0003, λ_s > 10), also

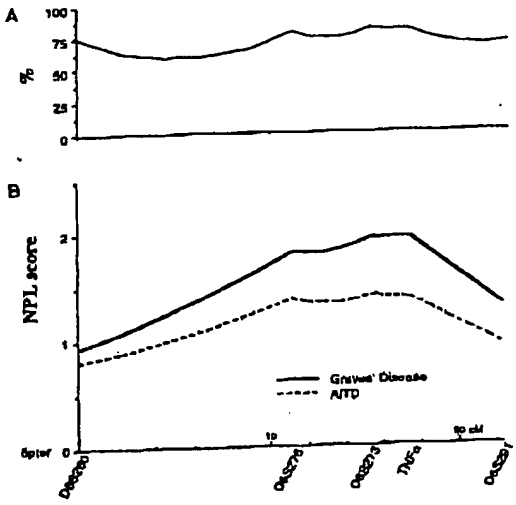

Figure 2. Multipoint linkage analysis of five markers spanning the *MHC* region. (A) Percentage information content over the marker map. (B) NPL scores obtained for Graves' disease (solid line) and AITD (dashed line) are shown against the marker order on the x-axis. The peak NPL score of 1.95 occurs at the markers *D6S273* and *TNFα*.

suggests that this polymorphism(s) may have a critical role in the susceptibility of males to GD.

Previous studies of the *CTLA4[AT]n* marker have not shown linkage to AITD; however, both these studies have employed a parametric method of linkage analysis which may be sensitive to mis-specification of background allele frequencies and the mode of inheritance (19,37). Furthermore, these studies combined families from a mixed ethnic background and used a more phenotypically varied population, with many families having only AH-affected subjects (19,37). Thus, our current study has important methodological differences from the *CTLA-4* linkage studies reported previously. Despite this, genuine differences in the contribution of the various susceptibility loci for GD are likely to exist between the UK and other populations, as has been already been demonstrated for the *CTLA-4* and other loci, in different IDDM populations (6–9,30,31).

In our population, *MHC* appears to have less influence on GD susceptibility than the *CTLA-4* region, with weaker evidence of linkage (NPL score 1.95, P = 0.026), and a locus-specific λ_s of 1.6 (Fig. 2). This contrasts with the findings in IDDM, where *MHC* has a consistently strong effect in all populations (6–9). However, our finding is not unexpected, as >95% of Caucasian IDDM subjects have a 'susceptible' IDDM *MHC* haplotype (*DR3/DR4*), compared with ~50% of control subjects, whereas only 50–60% of GD subjects carry the GD susceptible haplotype (*DR3*), compared with 20–30% of control subjects. Thus, *MHC* has a stronger influence on the development of IDDM than on GD. Our results also show that previous linkage studies of mixed AITD populations (mixed GD/AH families) (18,19) may have been unable to detect linkage of *MHC* to AITD due to allelic heterogeneity between GD and AH at this locus (10).

Using a multiplicative model, we can estimate that the *CTLA-4* locus (λ_s = 2.2) confers 29–34% of the total genetic susceptibility to GD in our population (38). It is therefore

unlikely that any other locus will have an effect on GD susceptibility that is stronger than that of *CTLA-4*. Our data also allow us to estimate, for the first time, that the *MHC* region ($\lambda_s = 1.6$) confers 17–20% of the genetic susceptibility to GD. Thus, taken together, these two loci account for ~50% of the inherited predisposition to GD in our population.

MATERIALS AND METHODS

Patients

Sixty-four families with two or more sibs affected with GD (including 146 with GD, 20 with AH and 72 unaffected subjects) were recruited from the north of England and the Lothian region of Scotland. GD was confirmed by the finding of biochemical hyperthyroidism, with evidence of one of the following: (i) significant TAO (American Thyroid Association Class 3 or worse) (39); (ii) diffuse increase in thyroid uptake on radionuclide scan; and (iii) positive serum thyrotropin-binding inhibitory immunoglobulin antibodies. The cohort of families comprised 53 full-sibs both with GD, five GD sib-trios, one GD quartet, five half-sibs with GD and six mixed GD/AH full-sibs (Table 1). Parents ($n = 49$) and unaffected sibs ($n = 36$) were studied wherever available. Additional second degree relatives had GD ($n = 4$), AH ($n = 3$) or were unaffected ($n = 5$). There were 122 female and 24 male GD patients, with a mean age at onset of 35.5 years (range 9–67 years). Fifty-four (37%) of the GD patients had significant TAO and two had thyroid dermopathy. All members of these sibships were Caucasian, and >95% of grandparents were of mainland UK or Irish origin. DNA from normal control subjects without evidence or family history of autoimmune disease were also obtained from the local population. All studies were carried out with the approval of the regional and district ethics committees.

Genotyping

The microsatellite markers were genotyped using fluorescently labelled PCR and resolved on a semi-automated 373 sequencer (Applied Biosystems, Foster City, CA). The primers, except for *CTLA4[AT]n*, were taken from the Genethon genetic linkage map (http://www.genethon.fr/genethon_en.html). The *CTLA4[AT]n* primers were: 5'-GCC AGT GAT GCT AAA GGT TG-3' and 5'-ACA CAA AAA CAT ACG TGG CTC-3'. Using these primers, the 112 mu allele of *CTLA4[AT]n* is equivalent to the 106 mu allele described previously (33). The marker map was derived from Genethon and the Southampton University database (http://cedar.genetics.soton.ac.uk/public_html/gmap.html). The *CTLA-4A/G* polymorphism in exon 1 of the *CTLA-4* gene was amplified using the following primers 5'-CCA CGG CTT CCT TTC TCG TA-3' and 5'-AGT CTC ACT CAC CTT TGC AG-3' followed by digestion with the restriction enzyme *Bst*71I (Promega, Southampton, UK) (32). The *Bst*71I digestion assay for the *CTLA4A/G* polymorphism was validated by direct DNA sequencing in five subjects, as described previously (40).

Statistical analysis

Two-point and multipoint NPL scores and marker information content were calculated using the 'score all' function of the GENEHUNTER package (41). The minimum proportion of full sib-pairs sharing zero alleles (z0) was calculated for each region using MAPMAKER/SIBS (42). Data from the chromosome 2 markers were weighted (0 or 1) for *CTLA4A/G* allele status using the modified GENEHUNTER-plus version 2 software, as described previously (43,44). The population allele frequencies for each marker were derived from local Caucasian controls. Family-based association analysis was performed on probands using an unaffected sib as an intrafamilial control (45), and were analysed using Fisher's exact test. *P*-values were Bonferroni corrected for multiple allelic comparisons, except for the markers *CTLA4A/G* and *CTLA4[AT]n*, where candidate alleles were known. ORs were calculated by Woolf's method (46).

ABBREVIATIONS

AH, autoimmune hypothyroidism; AITD, autoimmune thyroid disease; CI, confidence interval; CTLA-4, cytotoxic T lymphocyte antigen-4; GD, Graves' disease; HLA, human leukocyte antigen; IDDM, type 1 diabetes mellitus; MHC, major histocompatibility complex; mu, mobility units; NPL, non-parametric linkage; OR, odds ratio; TAO, thyroid-associated orbitopathy.

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